REMARKS

Claims 7, 24 and 25 are presently pending and under consideration. Claim 7 has been amended to recite that the sequence of the peptide consists of the amino acid sequence Cys Lys Pro Ile Ser Gly His Asn Ser Leu Phe Trp Tyr Arg Gln Thr (SEQ ID NO:1).

No new matter is added by this amendment to the claim 7.

1. Rejections under 35 U.S.C. § 112, First Paragraph

Claims 7, 24 and 25 are rejected under 35 U.S.C. § 112, first paragraph, allegedly, since the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention. According to the Examiner, it would require undue experimentation to practice the claimed invention for the reasons cited therein.

Applicants respectfully disagree. An invention meets the standard for successful practice set by Section 112 unless the invention is "totally incapable of achieving a useful result." *Brooktree v. Advances Micro Devices*, 24 U.S.P.Q.2D 1401, 1412 (Fed. Cir. 1992). The Examiner's attention is directed to the opinion of the Court of Appeals for the Federal Circuit (Federal Circuit) in *In re Brana*, 34 U.S.P.Q.2d 1437 (Fed. Cir. 1995). In *Brana*, the Board had affirmed a final rejection under Section 112, 1st paragraph, of claims covering certain compounds asserted to be useful as anti-tumor substances because it was alleged that the specification was non-enabling since it did not sufficiently establish that the claimed compounds had a practical utility, *i.e.*, as anti-tumor agents. 34 U.S.P.Q.2d at 1439.

The Federal Circuit emphatically reversed the Board's decision. The Federal Circuit explained the legal standard for compliance with the relevant Section 112 requirement, explaining that "unless there is reason to doubt the objective truth of the statements contained [in the specification] which must be relied on for enabling support", a specification's disclosure "must be taken as in compliance with the enabling requirement."

Id. at 1441 (emphasis in the original). Further, the *Brana* Court made clear that the Patent and Trademark Office has the initial burden of challenging a presumptively correct assertion of utility; evidence must be presented that those of skill in the art would doubt the disclosure. Only then must the applicant provide rebuttal evidence.

Further, the Federal Circuit in *Brana* explained that even if one of skill in the art would have questioned the asserted utility, all applicants need do to overcome the rejection is to proffer sufficient evidence to convince one skilled in the art of the asserted utility. *Id.* at 1441.

In the present invention, Applicants have provided such evidence. The Examiner's attention is again directed to U.S. Patent No. 5,911,990 to Marchalonis *et al.*, (Reference AA, of record), which clearly teaches that administration of the peptide of SEQ ID NO:1 to a mouse suffering from murine AIDS is able to restore normal levels of Th1 and Th2 cytokines (increase production of a Th1 cytokine and decrease levels of a Th2 cytokine) (see Abstract). Applicants note that the mouse model is an art accepted model for testing compounds for their effect in humans. The Examiner has provided no justification for dismissing the results of this study.

The Examiner's attention is also invited to Sepulveda *et al.*, 2003, J. Cardiovasc. Pharmacol. 41:489-497, ("Sepulveda I", Reference AF, of record). Sepulveda I also teaches that administration of the peptide of SEQ ID NO:1 to a mouse infected with LP-BM5 results in a longer progression time to mADIS by increasing production of at least one Th1 cytokine is increased or decreasing production of at least one Th2 cytokine. Further, in mice infected with both LP-BM5 and with coxsackievirus CVB3, which leads to myocarditis, Sepulveda I discloses that administration of the peptide of SEQ ID NO:1 provides a protective effect against the development of said myocarditis. See Sepulveda I, page 7, left column. Thus, a nexus between increasing production of at least one Th1 cytokine is increased or decreasing production of at least one Th2 cytokine and AIDS and/or

cardiovascular disease in an animal infected with an immunodeficiency-type retrovirus has been conclusively shown by Applicants. The Examiner has not provided a singe fact-based assertion that these results do not evidence enablement of the claimed invention.

The Examiner's attention is also invited to the manuscript of Sepulveda *et al.*, 2005, T-Cell Receptor Vβ8.1 Peptide Reduces Coxsackievirus-Induced Cariopathology in Aged Mice, Cardiovasc Toxicol. 5(1):21-8 ("Sepulveda II", which was submitted previously). Sepulveda II discloses that mice not infected with an immunodeficiency-type retrovirus, but infected with a coxsackievrus develop cardiopathology. However, administration of the peptide of SEQ ID NO:1 not only resulted in the increased production of at least one Th1 cytokine but also inhibited or reduced coxsackievirus-induced cardiopathology. Thus, in an animal free of an immunodeficiency-type retrovirus, the peptide of SEQ ID NO:1 increased production of at least one Th1 cytokine (IL-2), decreased production of at least one Th2 cytokine (IL-6) and provided a cardio-protective effect.

Finally, the Examiner's attention is invited to the manuscript of Yu *et al.*, entitled "A Role for T-lymphocytes in Mediating Cardiac Diastolic Function", which is in press in American Journal of Physiology (a copy of which is attached as Exhibit A hereto). Yu presents additional evidence administration of the peptide results in the increase in production of Th1 cytokine interferon-γ and a decrease in the production of Th2 cytokine IL-4. Further, the evidence shown in Yu confirms the findings of Sepulveda I and II, showing that administration of the peptide recited in claim 7 results in a cardio-protective effect.

Applicants do not understand why the Examiner has not taken note of these post-filing references. Applicants note that post-filing date references can be used to address the accuracy of a statement made in the specification, *i.e.*, that administering the peptide of SEQ ID NO:1 results in the increased production of at least one Th1 cytokine and/or decreased production of at least one Th2 cytokine. *Application of Marzocchi*, 439 F.2d 220, 169 U.S.P.Q. 367 (CCPA, 1971), fn. 4. Further, Applicant points out that the compositions

and methods used in the post-filing references were available at the time of filing, thus, conclusively proving that the specification as filed enables the claimed method. Moreover, the experiments were performed in art-accepted animal models. Applicants further note that the Examiner has not provided any fact-based evidence that is specific to the claimed invention to doubt that these post-filing date references show that the claimed invention is enabled as originally filed. Applicants assert that the Examiner's statements regarding the invention must focus on the claimed invention and not on the field of peptide-based drugs in general.

As to the Examiner's concerns about the alleged scientific deficiencies of the specification set forth on pages 4-5 of the Office Action dated March 25, 2004, Applicants submit that the Examiner has mistaken his role as an examiner in the Patent and Trademark Office for that of an examiner at the Food and Drug Administration. The Examiner is again reminded of the Federal Circuit's admonition in *In re Brana* that testing for the full safety and effectiveness of a product is more properly left to the Food and Drug Administration and the requirements under the law for obtaining a patent should not be confused with the requirements for obtaining government approval to market a particular drug for consumption. *Id.* at 1442; *see*, *Scott v. Finney*, 34 F.3d 1058, 1063 (Fed. Cir. 1994). As stated in *Scott*, "[t]esting for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings."

Further, we note that none of the scientific deficiencies mentioned by the Examiner have any bearing on the enablement of the invention as claimed under 35 U.S.C. § 112. For instance, whether an immune response would be elicited against the peptide is immaterial as to whether the peptide can be administered to modulate immune function in accordance with the teaching of the specification. Nevertheless, Applicants have shown that the peptide can be so administered and that immune function is modulated as claimed in an *in*

vivo setting in an art-accepted animal model. Applicants also note that the Examiner on page 8 of the Office Action has actually requested data showing that the claimed peptide or derivatives thereof were capable of modulating immune response in the desired manner. Such results have already been presented in previous responses and are presented herein again, *supra.*

Further, addressing the Examiner's point on page 4 of the Office Action that the claims encompass numerous species of peptides, Applicants note that the peptide administered consists of the amino acid sequence of SEQ ID NO:1. No derivatives of the peptide are encompassed in the claims. Thus, there is no ambiguity in the identity of the peptide being administered.

In view of the above, it is submitted that the specification provides sufficient teaching to allow one skilled in the art to successfully make and use the claimed methods for increasing the production of at least one Th1 cytokine or to decrease the production of at least one Th2 cytokine, without undue experimentation. The rejection under Section 112, first paragraph, therefore, should be withdrawn.

CONCLUSION

Applicants respectfully request that the above-made remarks of the present response be entered and made of record in the file history present application.

Applicants request that the Examiner call Thomas E. Friebel at (212) 326-3811 if any questions or issues remain.

Respectfully submitted,

Date: April 27, 2005

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Enclosures

A Role for T-lymphocytes in Mediating Cardiac Diastolic Function

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ABSTRACT

Induction of T-helper (TH) lymphocyte by distinct TH ligands results in a differentiation to TH1/TH2 subsets based on their unique pattern of cytokine secretion and effector functions. We hypothesized that the relative proportion of TH1/TH2 directly relates to cardiac fibroblast (CF) function and thereby cardiac extracellular matrix (ECM) composition and cardiac diastolic function in the absence of injury or altered wall stress. We compared the effect of selective TH1 with TH2 inducers on cardiac gene expression, ECM composition, and diastolic function in C57BL/J mice. Twelve weeks after immune modulation, the left ventricular stiffness (β) was significantly increased in the TH1 mice and decreased in the TH2 group ($p<0.01$). The TH2 group also demonstrated significantly increased end-diastolic and end-systolic volumes ($p<0.01$). Cardiac gene expression pattern for pro-*MMP-9* and *-13* were increased by greater than five-fold in the TH2 group and significantly decreased in the TH1 group ($p <0.05$). The total cardiac collagen and crosslinked collagen were significantly increased in the TH1 and decreased in the TH2 ($p <0.01$). Co-culturing lymphocytes harvested from the treated mice with naive primary CF demonstrated a direct control of the lymphocytes on CF pro-collagen, pro-MMP gene expression and MMP activity. These results suggest that TH phenotype differentially affects diastolic function through modulating CF *pro-collagen* and *pro-MMP* gene expression, MMP activity and cardiac collagen crosslinking, resulting in altered ECM composition. Thus, modulation of the TH lymphocyte function could promote adaptive remodeling in heart failure and post-myocardial infarction.

Key Words: LP-BM5 retrovirus; T-helper cell, cardiac fibroblast; extracellular matrix; Collagen Types I and III; TH1, TH2, Crosslinking, T-cell receptor peptide

INTRODUCTION

Diastolic heart failure recently has been reported to have a mortality rate of 23% during a 3.1 year follow-up with optimized medical therapy (23). A central contributory factor in diastolic dysfunction is maladaptive remodeling of the extracellular matrix (ECM), whose composition is controlled and maintained by the cardiac fibroblasts (1; 7; 13; 16). T-lymphocyte infiltration of post-myocardial infarction coincides with increased cardiac fibroblast proliferation and fibrotic function (57) which is linked to the notion that lymphocytes are essential in the wound-healing process (2; 3). The cooperation of the T-lymphocyte with fibroblasts in cardiac remodeling secondary to tissue injury is therefore a predictable concept. Correspondingly, we propose that alteration of T-lymphocyte function may affect cardiac ECM composition, thus diastolic function, in the absence of cardiac tissue injury or increased wall stress.

Cardiac fibrillar collagen homeostasis is mediated by cardiac fibroblasts via a balance of synthesis, degradation and cross-linking (16). The primary forms of synthesized collagen in the heart are collagen type I (Col I), making up 85 percent of the total collagen, followed by 11 percent of collagen type IIII (Col III). Secondly, the Col I and Col III degradation pathway is mediated by collagenases, MMP-1, -8 and -13 and gelatinases MMP-2 and -9 (43). Thirdly, collagen crosslinking has been demonstrated to alter ventricular compliance and may affect collagen degradation (1). Thus, any imbalance among collagen biosynthesis, degradation and crosslinking may contribute to left ventricular diastolic dysfunction (6). Hence, many lines of evidence support that cardiac

ECM composition correlates with cardiac mechanical function. However, there is little direct evidence that integrates lymphocyte and cardiac fibroblast functions with ECM composition.

The differentiation process of naive T-helper lymphocytes (TH) leads to the generation of effector cells, namely TH1 and TH2 cells, where TH1 are characterized by the cytokines IL-2, -12, -15, -18, IFN-γ and TNF-β expression and TH2 by IL-4, -5, -6, -10, -13 and -17 (39). The lymphocyte recently has been shown to modulate fibroblast collagen formation in non-cardiac tissues, namely: hepatic, dermal, pulmonary and synovial (8; 9; 21; 34). Moreover, decreased cardiac and vascular compliance in pre-eclampsia, angiotensin II-induced hypertension, and spontaneously hypertensive rats have been associated with TH1 cytokine levels (36; 41; 51). Agents that suppress T lymphocyte function, such as steroids (10), retinoic acid, sirolimus (18) and cyclosporine A (15), markedly impair wound healing. In liver transplantation patients treated with either cyclosporine A or tacrolimus immunosuppression there is a significant deterioration of diastolic without changes in systolic function after three months of immunosuppression (45). Rodent studies support this observation where markedly altered cardiac ECM composition occurred with cyclosporine at clinical immunosuppressive doses (14; 38). Pauschinger et al demonstrated significantly increased Col I and Col III ratios in dilated cardiomyopathy (DCM) but could not detect the profibrotic cytokines TGF-β_1 or TGF-β_2 in human DCM heart biopsies (37). This report suggested that other cytokines may be involved in the regulation of cardiac fibroblast collagen production in the heart. Clinical data

support this conclusion, as the balance of TH1 and TH2 cytokine gene expression is directly related to the mortality rate of DCM with heart failure (46).

In view of the importance of cytokines in heart disease, the present study determined the effect of selective modulation of lymphocyte function on the ventricular function. Susceptible mice such as the C57BL/6 strain infected with the LP-BM5 variant of murine leukemia virus have a deficient immune response correlated with a decreased TH1/TH2 ratio, but TH1 activity that can be restored by administration of peptides derived from the variable segment of T-cell receptor Vβ chains (56). Our results revealed that the TH2 immune condition significantly alters the cardiac ECM and cardiac diastolic function, whereas TH1 leads to profibrotic activity and increased ventricular stiffness. This investigation of lymphocyte-directed cardiac diastolic function is in the absence of cardiac tissue inflammation, cellular infiltration or injury.

MATERIALS AND METHODS

Mice

Four-week-old female C57BL/J6 mice were obtained from Charles River Laboratories (Wilmington, DE, USA). During the study the mice were maintained in the facility on an NIH-31-modified mouse sterilized diet (mouse diet #7001; Teklad, Madison, WI, USA) and water *ad libitum*. This study was approved by the animal review committee. The procedures in the "*Guidelines for the Care and Use of Laboratory Animals*" (DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205) and "*Principles of Laboratory Animal Care*" (published by the National Society for

Medical Research) were followed in this study. Mice were randomly assigned to one of the following Groups: **I**, uninfected control mice; **II**, mice infected with LP-BM5 retrovirus; **III**, uninfected mice given TCR Vβ peptides 5.2 and 8.1; and **IV**, mice infected with retrovirus and vaccinated with TCR Vβ peptides 5.2 and 8.1.

LP-BM5 Viral Infection

LP-BM5 retrovirus mixture was administered intraperitoneally to mice in 0.1 mL of minimum essential medium with an esotropic titer of 4.5 $\log_{10}$ plaque forming units x 10^{-3}/L, which induces TH2 dominant lymphocyte function comparable with that previously published (17; 20; 47; 58). The mice were infected with LP-BM5 two weeks before treatment with the TCRVβ peptides as done previously in earlier studies (27; 40; 49). Hemodynamic studies and tissue acquisition were performed 12 weeks post-induction of TH2 immune function with the LP-BM5 mixture.

T-cell Receptor Vβ Peptides

The TCR Vβ peptides corresponding to the CDR1 segments of human Vβ 5.2 and V 8.1 at a dose of 200 μg/mouse and vehicle control were administered i.p. on days 14 and 28 in the non-infected and post-LP-BM5 infection mice. The sequences of these TCR Vβ peptides were C K P I S G H N S L F W Y R Q T (Vβ 8.1) and C S P K S G H D T V S W Y Q Q A (Vβ 5.2) and manufactured by Minotopes Clayton, Victoria, Australia. Hemodynamic studies and tissue acquisition were performed 12 weeks post-induction of TH1 immune function.

Quantification of left ventricular mechanics

The Millar Conductance Catheter System (CCS) was used, as has been previously described by our laboratory (53-56). All mice were anesthetized with urethane in saline (1000 mg/Kg, i.p.) and α-chloralose in propylene glycol (50 mg/Kg, i.p.). This anesthetic technique causes minimal cardiac and vascular depression and inhibits CNS catecholamine outflow that may confound data interpretation (11). The mice were ventilated and the external jugular vein cannulated for volume administration. The apical portion of the heart and the inferior vena cava (IVC) were exposed through a substernal-transverse incision and a Millar Conductance Catheter 1.4 Fr (Millar Corporation, Houston, TX) was inserted into the apex of the left ventricle. Pressure-volume loops were acquired and computed as previously reported (56).

RNA Extraction, RT-PCR and Real-Time PCR

Cardiac tissues were processed according to the methods described by Yu, Larson et al (59). Cardiac and lymphoid tissues were harvested in TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Diluted cDNA was used for reaction with Qauntitec Sybr green PCR kit (Qiagen) 50 µl, and real-time PCR RT-PCR was performed by monitoring the increase of fluorescence of SYBR Green using the Rotor-Gene RG-3000 (Corbett Research) in a 72-well rotor. Custom primers were designed using the Integrated DNA Technologies. Primer quality (lack of primer-dimer amplification) was confirmed by melting curve analysis. In addition, gel-electrophoresis of each primer-product was shown to have only a single band. In each experiment, the relative amounts of mRNA for target genes were calculated from the standard curves and normalized to the

relative amounts of reference gene RNA (β-actin mRNA), which were obtained from a similar standard curve. Real time PCR primers are listed in Table 1.

The method for RT-PCR was used to determine the gene expression of *Col Iα2* and *MMP-13* of cultured cardiac fibroblasts when co-cultured with lymphocytes as described by our previous report (59). The *Col Iα2* (sense) 5'-GCGTACCTGGATGAGGAGAC-3' (antisense) 5'-GTCTTGCCCCATTACATTTGT-3' and *pro-MMP-13* (sense) 5'-GAT GAC CTG TCT GAG GAA GAC C-3' (antisense) 5'-GTC ACA TCA GAC CAG ACC TTG A-3' were normalized with 18s rRNA QuantumRNA® (Ambion Inc. Austin, TX USA).

Cardiac fibroblast and lymphocyte isolation for co-culture

An isolation protocol provided by Thomas Borg, Ph.D. (University of South Carolina), yields a cardiac fibroblasts purity of >88% using the cardiac fibroblast specific DDR2 antibody with FACS. In brief, ventricular sections were minced and sequentially digested 15 times with 0.54 mg Liberase 3 enzyme (Roche Biochemical) in 60 mL warm sterile Krebs-Henslett buffer and the adherent fibroblasts were plated in a T75 with 20 mL of DMEM/Hepes with 10% fetal bovine serum medium. These primary fibroblast cultures were grown to 80% confluence and the fetal bovine serum decreased to 1% prior to co-culturing with lymphocytes.

The spleen was removed with sterile technique, sliced, and forced through a 30 mesh screen filter. The splenic lymphocytes were isolated with lymphocyte Separation Medium (Cellgro, Herndon, VA) and washed three times with RPMI

culture media. The lymphocytes from the individual treatment groups were added directly to the fibroblast cultures at a concentration of 5 x 10^5/mL. After 48 h the lymphocytes were removed by washing and the adherent cardiac fibroblast removed for RNA extraction and RT-PCR and the supernatant used for zymography.

Zymography

Gelatin zymography was performed using lymphocyte-fibroblast co-culture supernatants. Briefly, culture medium (15 μL) was diluted 1:1 with SDS loading buffer and applied to precast 10% polyacrylamide gel Zymogram (Novex, Frankfurt, Germany). Following electrophoresis at 100 V, 4°C, for 120 minutes, the gels were washed twice with renaturing buffer at room temperature for 30 min. Zymograms were then transferred into activity buffer and developed at 37°C for 12 h. Following fixation and staining with Coomassie Brilliant Blue G-250 (0.25%), the zymograms were de-stained with 10% (vol/vol) acetic acid and bands quantified image analysis (BIO-RAD GS-800).

Determination of hydroxyproline and collagen crosslinking. Hydroxyproline, an amino acid found exclusively in connective tissues, is used as a means to quantify collagen. Each left ventricle was separated into two samples, dried, and weighed for tissue analysis. One sample was analyzed for total myocardial hydroxyproline after acid hydrolysis according to the methods of Stegeman and Stadler (44). The second sample was extracted and digested with (CNBr) digestion according to Woodiwiss et al (52), which is a modification of that originally described by (35). Percent crosslinking was determined by comparing

the total hydroxyproline with CNBr-soluble hydroxyproline. The data were expressed as microgram of collagen per milligram of dry heart weight, assuming that collagen contains an average of 13.5% hydroxyproline.

Statistical Analysis

ANOVA with multi-comparison procedures was used to test the differences among the defined groups with SPSS version 11.5. Values obtained from treatment groups were compared with control values using the Student's t-test. Comparable non-parametric tests (Kruskal-Wallis and the rank sum test) were substituted when tests for normality and equal variance failed. All data are reported as means ± standard error of the mean.

RESULTS

Cardiovascular function relative to immune function. Figure 1 illustrates the effect of immunomodulation on the acquired pressure-volume loops of the four groups. The immunological features of LP-BM5 infection are splenomegaly and a skewing to the T-lymphocyte function to TH2. Table 2 shows a 14-fold increase ($p<0.001$) in spleen weight to body weight ratios in the LP-BM5 groups twelve weeks after infection which is consistent with that previously described by Watson et al (28; 40; 48). The fundamental TH2 cytokines, IL-4 and IL-10, increased by five-fold ($p=0.008$) and the TH1 cytokine, IFN-γ, decreased by 2.1-fold ($p=0.01$) compared with controls (Table 3A). Table 2 shows that the left ventricular function of the LP-BM5 treatment group demonstrated a 61% decreased ventricular stiffness, β, ($p=0.0009$) and ventricular dilation described by a 28% increase in left ventricular end-diastolic volume, Ved, ($p=0.0009$) and a

43% increase in left ventricular end-systolic volume, Ves, ($p=0.00004$) compared with the control group. The pre-load and afterload independent parameters describing isovolumic contraction and relaxation, dP/dt_{max}-Ved and τ Weiss respectively, also were significantly prolonged ($p<0.01$) in the infected group compared with the controls. The SVI and CI increased by 43% and 25%, respectively, in the infected mice, which may have been related to a 34% decrease in afterload, Ea ($p=0.0008$). Therefore, the LP-BM5 treatment induced a TH2 lymphocyte phenotype that coincided with decreased diastolic and systolic function without a reduction in cardiac output.

With selective TH1 stimulation by means of TCR Vβ5.2 and 8.1peptides, β increased without affecting any other parameter compared with the control group. Confirmation of TH1 stimulation is supported by a 12-fold increase in IFN-γ and a 3.5-fold decrease in IL-4 (Table 3A). However, combining LP-BM5 with TCR peptide treatments the Ved, Ves, β, dV/dt_{min} – Ved, and Ea did not differ when compared with control group (Table 2). This combination of treatments has been well-documented to modulate lymphocyte secretory function (29; 40). Therefore, TH1 is associated with increased left ventricular stiffness and also reversal of TH2-mediated decrease in left ventricular stiffness and ventricular dilation.

Alterations of cardiac pro-collagen and pro-MMP mRNA with T-lymphocyte polarization. To compare the cardiac mRNA expression, real-time PCR analysis was carried out using the total RNA isolated from the mid-portion of the left ventricle. Important in the context of pro-collagen and pro-MMP gene

expression, the cardiac ACE and TGF-β_1 gene expression was not different from the control group in these treatment groups (data not shown). However, Table 3B demonstrates that the LP-BM5 group had a six-fold decrease in cardiac *pro-Col Iα2* expression and up-regulation of pro-*MMP-9* and *pro-MMP-13* gene expressions. Especially striking was the five-fold reduction of pro-MMP-9 and -13 gene expression in the cardiac tissues with TCR peptide stimulation of TH1 function. The combined treatments resulted in a reduced *pro-Col Iα2* expression that was not different from the LP-BM5 group and a similar suppression of the MMPs as seen in the TCR peptide group. These data suggest that there is a separation in the immune regulation of collagen synthetic from degradative genes, namely: TH1 immune function relates to MMP gene expression and TH2 to collagen gene expression.

Cardiac collagen content and crosslinking. We sought to determine whether the difference in ventricular stiffness relative to the immune condition was associated with the total collagen and/or the level of crosslinking. The total collagen (Figure 2), as determined with the hydroxyproline method, decreased by 23% ($p=0.005$) in the LP-BM5 group and increased by 34% ($p=0.03$) within the TCR peptide group compared with the control group. The correlation of total cardiac collagen versus ventricular stiffness (β) was found to be $R^2=0.409$ ($p=0.01$).

To further evaluate the involvement of collagen crosslinking related to immunomodulation and ventricular stiffness, we compared the measured

collagen crosslinking in response to immune modulation. The LP-BM5 group revealed a markedly decreased collagen crosslinking by 4.8-fold ($p = 0.001$) and TCR peptide treatment resulted in a 67% ($p = 0.03$) increased collagen crosslinking (Figure 3). A realistic interpretation is that, since the MMP expression was significantly altered with the treatments, these crosslinking results represent a cumulative effect due to the three-month treatment period. Most importantly, the relationship between collagen crosslinking and ventricular stiffness (Figure 4) with a $R^2=0.616$ ($p=0.005$) reveals that this collagen maturation step is an essential determinant of the filling phase of diastole.

Co-culturing of lymphocytes with primary cardiac fibroblasts. To determine if the observed changes in cardiac gene expression were a direct or indirect effect of the lymphocyte phenotype, we applied lymphocytes isolated from the treated mice to primary cardiac fibroblast cultures harvested from non-treated C57BL/J6 mice. Lymphocytes from the control, LP-BM5, and LP-BM5 plus TCR peptide groups were pooled related to the treatment groups and co-incubated with primary cardiac fibroblasts. Figure 5 shows that *pro-collagen Iα2* and *pro-MMP-13* cardiac fibroblast gene expression are directly related to the induced lymphocyte function. The LP-BM5 treatment alone caused a 30% decrease in *pro-collagen Iα2* and a 13-fold increase in *pro-MMP-13* cardiac fibroblast gene expression. The addition of TCR peptide treatment to the LP-BM5 group resulted in a six-fold increase in *pro-collagen Iα2* and a two-fold decrease in *pro-MMP-13* gene expression. Figure 6 shows the zymography of the supernatant taken from the co-culture at 48H. The TCR peptide induced a decrease in

MMP-9 active form (p=.0.04), while LP-BM5 significantly increased MMP-9 latent form (p=0.02). Even though *in vitro* effects were after 48 h of co-culture – whereas the *in vivo* results were after three months – this co-culture model provides supportive evidence for the *in vivo* experiments, suggesting that the lymphocyte phenotype directly corresponds to cardiac fibroblast function.

DISCUSSION

The present study demonstrates that skewing the immune response to either TH1 or TH2 markedly affects systolic and diastolic ventricular function. This immunological modification of ventricular function coincides with MMP and collagen gene expression, myocardial collagen content and collagen crosslinking. The selective induction of TH1 lymphocytes, accomplished by using the T-cell receptor peptides Vβ 5.2 and 8.1, increased ventricular stiffness – perhaps through decreased MMP activity and increased collagen synthesis and crosslinking. Conversely, enhancement of TH2 with LP-BM5 retrovirus resulted in ventricular dilation and decreased ventricular stiffness through enhanced MMP activity and decreased collagen synthesis and crosslinking. The TH2-induced diastolic dysfunction was reversed by concurrent enhancement of the TH1 immune response. However, the selected lymphocyte effects of the LP-BM5 retrovirus on TH2 induction is not reversed by the TH1 TCR peptide treatment – as evidenced by the splenic weights and IL-4 and IFN-γ gene expression. Therefore, there appears to be a simultaneous induction of the TH1 and TH2 lymphocyte populations in the combined treatment group. Our results suggest,

therefore, that genes affected by TH2 remain elevated together with those affected by TH1. Essentially, this parallel induction of TH1 and TH2 lymphocyte functions results in the normalization of cardiac function.

LP-BM5 and TCR peptides

The T-cell receptor Vβ peptides 5.2 and 8.1 are specific to the T-lymphocyte, do not directly affect fibroblast function or other organ systems, and have been demonstrated to significantly induce TH1 lymphocyte cytokines (29; 31; 32; 40). Support for using T-cell receptor peptides in this investigation is described by Kessels et al, who proposed the T-cell receptor peptides as a viable therapeutic for induction of virus or tumor-specific immunity due to their specificity in redirecting T-cell immunity (25). Similarly, the LP-BM5 retrovirus infection of lymphocytes is selective due to the administered virus mixture containing murine leukemia retrovirus (MuLV) complex of BM5def and BM5eco viruses. The BM5def virus is the causative agent for the induction of immunodeficiency and the BM5eco is necessary for the virus propagation and thus functions as a helper virus. The dependency of the BM5def on the BM5eco for lymphocyte infection and viral replication results in only one round of viral replication. To exclude the possibility of retrovirus infection of cardiac fibroblasts, we co-incubated LP-BM5 with primary cardiac fibroblast cultures. We found no significant increase of LP-BM5 copy numbers as detected by cPCR over time (data not shown). The LP-BM5-induced TH2 lymphocyte function is rapid and sustained until the death of the mouse (17; 20; 28; 47; 58). We have reported ventricular dilation and decreased ventricular stiffness occurs in the absence of

cardiac lymphocytic infiltrates and inflammatory mediators (4; 40). Moreover, we have not been able to demonstrate iNOS or TNF-α over-expression in the cardiac tissues of LP-BM5-infected mice (4). We have previously demonstrated an increased intracellular space which may account for the ventricular dilation that has been hypothesized to result from the increased production of hydrophilic glycosaminoglycans (4; 50). Therefore, it can be concluded that the T-cell receptor peptide and the LP-BM5 murine model do not induce obvious myocardial pathology, infection or inflammation. This, therefore, supports the conclusion that T-cell receptor peptide and LP-BM5-induced TH1 and TH2 related function lymphocyte are the mediators of the reported hemodynamic and ECM changes.

Extracellular matrix

The extracellular factors affecting passive ventricular tension include collagen characteristics attributable to content, type and crosslinking in addition to collagen coupling to the adhesion molecules via the Z-band (5). When there is a significant change in myocardial collagen fraction, the diastolic function is compromised, which is supported by our correlation of total collagen with ventricular stiffness. Cardiac fibroblasts are ubiquitous cardiac cells providing mechanical strength to the tissue by secreting extracellular matrix components that define the composition and function of the cardiac ECM (7) (22; 50). A reduction in the number of fibrillar collagen tethers allows for myocardial dilation

and slippage of myocytes (50), which may account for the observed significantly increased Ved and Ves in the LP-BM5 treatment group.

The accepted regulatory factors of the cardiac ECM collagen include angiotensin II (Ang II), transforming growth factor-β_1 (TGF-β_1), connective tissue growth factor (CTGF), osteopontin (OPN) and endothelin-1 (ET-1) (reviewed Dostal et al (12). In summary, we found no change in gene expression for *ACE* and *TGF-β1*. However, the *pro-Col Iα2* and *pro- MMP*s genes were remarkably affected by immunomodulation, with the pro-MMP genes being oppositely altered by TH1 and TH2 conditions and the *pro-Col Iα2* with only TH2.

Collagen crosslinking

The level of collagen crosslinking detected with immunomodulation parallels that of diastolic stiffness. Human cardiac tissues demonstrate left ventricular dilation with normal and increased total cardiac collagen concentrations (19). Collagen crosslinking has, therefore, been shown to be the primary determinant of myocardial stiffness and dilation (1; 52). Kato demonstrated that selective inhibition of cardiac collagen crosslinking with six weeks of treatment with β-aminopropionitrile markedly decreased ventricular stiffness and increased end-diastolic volume (24). We found that the relative abundance of ventricular collagen crosslinking also is associated with ventricular stiffness and, additionally, the crosslinking is increased in our TH1 treatment but decreased with TH2 immune function. The MMP gene expression was particularly high in the TH2 mice – it therefore is likely that the collagen turnover was

correspondingly high and thereby possibly affecting the collagen maturation process related to crosslinking as suggested by Gunja-Smith et al (19). These observations collectively support that passive diastolic function is related to cardiac collagen crosslinking and crosslinking is affected by immune function.

MMP and Immune function

Consistent with our report, IL-4 has been shown to induce MMP-9 in the absence of IFN-γ (42). Cardiac fibroblasts are the primary cells involved in cardiac remodeling, including synthesizing ECM collagen and MMPs (30). They are also a major source of TGF-β_1 and Ang II, which are primary stimulatory factors for cardiac fibroblast function related to collagen and MMP synthesis. However, we report that TGF-β_1 and ACE were not over-expressed in our model of immunomodulation. This does not exclude non-cardiac sources for these secretory factors. Given that our model did not incorporate myocardial injury, the role of pro-inflammatory cytokines is not expected to contribute to the induction of MMPs, especially since our previous studies failed to identify iNOS and TNF-α in the cardiac tissues following LP-BM5 (4). Our data also support that of others suggesting the chronic loss of interstitial collagen is associated with both systolic and diastolic function (26). It can be concluded that TH2 cytokines induced MMPs in our cardiac tissues that were significantly inhibited by TH1 cytokines with a parallel change in ventricular function.

Integration

The challenging aspect in defining the role of the lymphocyte TH1/TH2 paradigm in remodeling of the cardiovascular ECM matrix is the integration of this system

with the neurohormonal pathways that are well characterized as regulators of cardiovascular remodeling. It is known that inhibition of the TH1 function prevents cardiac remodeling in Ang II-treated mice (33), whereas, Ang II enhances TH1 via AT1 receptors (41). Accordingly, our *in vivo* findings do not exclude the neurohormonal and wall stress contributions to cardiovascular ECM remodeling but suggest that the lymphocyte is also a major contributor in the overall process. However, our *in vitro* studies provide persuasive evidence that there is a direct and differential effect of polarized TH1 and TH2 lymphocytes on cardiac fibroblast gene expression and function. It is accepted that a pathological increase in neurohormonal or wall stress induces remodeling and we propose that in a similar manner an imbalance in TH1/TH2 function may contribute and direct the overall cardiovascular remodeling process. Therefore, we have selected a model that minimizes the neurohormonal and wall stress components, namely LP-BM5 and TCR peptides. The study contained herein suggests that altering T-lymphocyte function may induce changes in the cardiac fibroblast function that could provide a means to manipulate the remodeling processes resulting in optimized cardiac function. We have provided support for this concept through selective inducers of lymphocyte TH1/TH2 function. Therefore, immunomodulation of T-lymphocyte function for treatment of cardiovascular disease is a concept that may have promising clinical significance. In the future, we plan to demonstrate that modulation of the immune function may have a significant therapeutic effect on pathological remodeling caused by neurohormonal and/or wall stress pathways.

ACKNOWLEDGEMENTS

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Table 1.

	Real Time PCR	
Primers/ Accession #	**Forward/ Bases**	**Reverse/ Bases**
β-actin BC040513	5'-TTGCTGACAGGATGCAGA AG-3' 204 - 223	5'-TGA TCC ACA TCT GCT GGA AG-3' 350- 331
Pro-Col Iα2 NM_007743	5'-GCGTACCTGGATGAGGAGAC-3' 3929 - 3948	5'-GTCTTGCCCCATTCATTTGA-3' 4080 -4062
Pro-Col IIIα1 AK079113	5'-TTCTGCTGTTGCTGGTGAAC-3' 1631 -11650	5'-TGGCTTGAATGAAGGTACCAA-3' 1749 - 1729
Pro-MMP-13 NM_008607	5'-GATGACCTGTCTGAGGAAGACC -3' 98-119	5'-GTCACATCAGACCAGACCTTGA -3' 459 -438
Pro-MMP-9 NM_013599	5'-CTTCGACACTGACAAGAAGTGG -3' 1140 -1161	5'-CTTGTAATGGGCTTCCTCTAT -3' 1712 - 1694
IL-4 NM_021283	5'-CCACCATGAATGAGTCCAAG-3' 429 -447	5'-AAGTTAAAGCATGGTGGCTCA-3' 534 -514
IFN-γ NM_008337	5'-CTGCTGATGGGAGGAGATGT-3' 969 -988	5'-GGAAGCACCAGGTGTCAAGT-3' 1105 -1086
IL-10 NM_010548	5'-CCAGGGAGATCCTTTGATGA-3' 1083 -1102	5'-GCTGCTACAAAGGCAGACAA-3' 1284 -1265

TABLE 2.

Groups		I	II	III	IV
Treatment		Control	LP-BM5	TCR	LP-BM5 + TCR
n		20	9	20	9
Age	mo	4	4	4	4
BW	g	35.2 ± 1.3	30.1 ± 0.8[a]	33.4 ± 1.6	27.0 ± 0.82[ac]
Spleen/BW	mg/g	4.2 ± 0.6	61.8 ± 2.8[b]	4.2 ± 0.6[d]	57.8 ± 7.2[b]
Left Ventricular and Aortic Functional Parameters					
HR	BPM	552 ± 11	483 ± 18[b]	518 ± 25	498 ± 36
SVI	µL/g	0.37 ± 0.02	0.53 ± 0.07[b]	0.40 ± 0.03	0.47 ± 0.03[b]
EF	%	55.8 ± 2.6	46.4 ± 3.1[a]	58.8 ± 3.0[d]	57.3 ± 5.3
CI	µL/min/g	204 ± 11	254 ± 36	207 ± 16	237 ± 22
SWI	mmHg.µL/g	28.1 ± 1.6	31.9 ± 4.6	28.3 ± 1.5	31.6 ± 2.0
β	mmHg/µL	0.18 ± 0.02	0.07 ± 0.02[b]	0.27 ± 0.02[b]	0.23 ± 0.05[d]
Ved	µL	23.8 ± 1.0	33.1 ± 2.9[b]	21.4 ± 1.1	23.6 ± 3.1[c]
Ves	µL	11.0 ± 0.7	19.4 ± 1.5[b]	9.8 ± 0.9	11.7 ± 2.7[c]
dP/dt_{max}	mmHg/s	9095 ± 454	6849 ± 827[a]	8527 ± 622	7848 ± 994
dP/dt_{min}	mmHg/s	-6711 ± 369	-5024 ± 606[a]	-6275 ± 372	-5375 ± 417[a]
PRSW	mmHg	104 ± 3	72 ± 3[b]	98 ± 4[d]	93 ± 5[d]
dP/dt_{max}-Ved	mmHg/µL	565 ± 26	327 ± 33[b]	732 ± 207[c]	670 ± 94
Tau weiss	ms	6.0 ± 0.3	8.1 ± 0.6[b]	6.4 ± 0.3[d]	7.2 ± 0.6
Ea	mmHg/µL	6.8 ± 0.3	4.5 ± 0.5[b]	6.4 ± 0.7	5.6 ± 0.4[a]

Table 3.

A

Splenic Tissue	I	II	III	IV
	Control	LP-BM5	TCR	LP-BM5 + TCR
IL-4	1	5.1[b]	-3.5[a]	4.2[b]
IL-10	1	5.3[b]	ND	1.8[c]
IFN-γ	1	-2.1[b]	12.6[bd]	2.0[d]

B

Heart Tissue	I	II	III	IV
	Control	LP-BM5	TCR	LP-BM5 + TCR
β-actin	1	1	1	1
Pro-Col Iα2	1	-6.4[b]	-0.6[d]	-6.2[b]
Pro-Col IIIα1	1	2.1	-2.3[ac]	-0.5[c]
Pro-MMP-9	1	6.7[b]	-5.2[a]	-0.5[c]
Pro-MMP-13	1	2.9[a]	-5.4[bc]	-4.7[ac]

Figure 1.

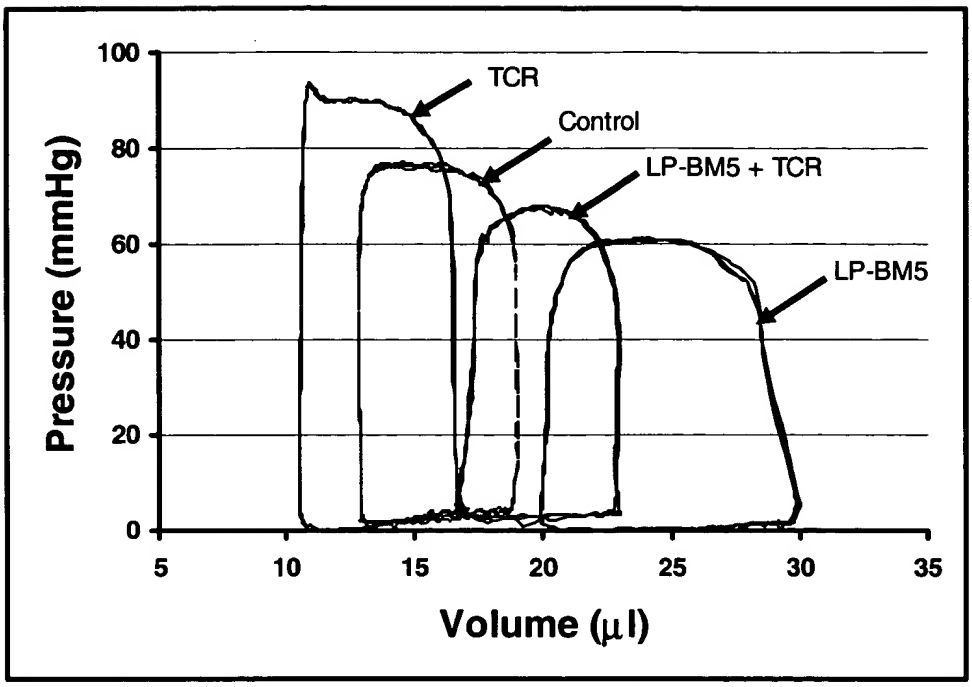
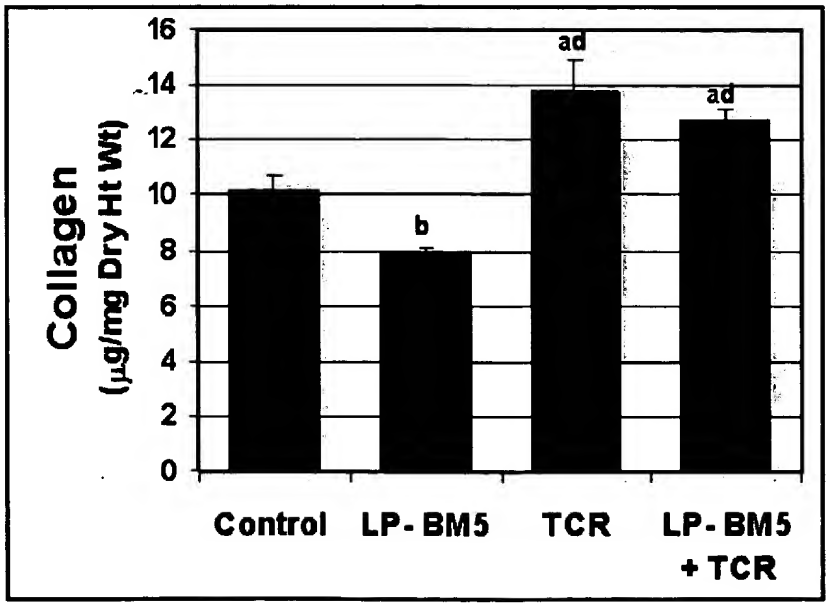


Figure 2.



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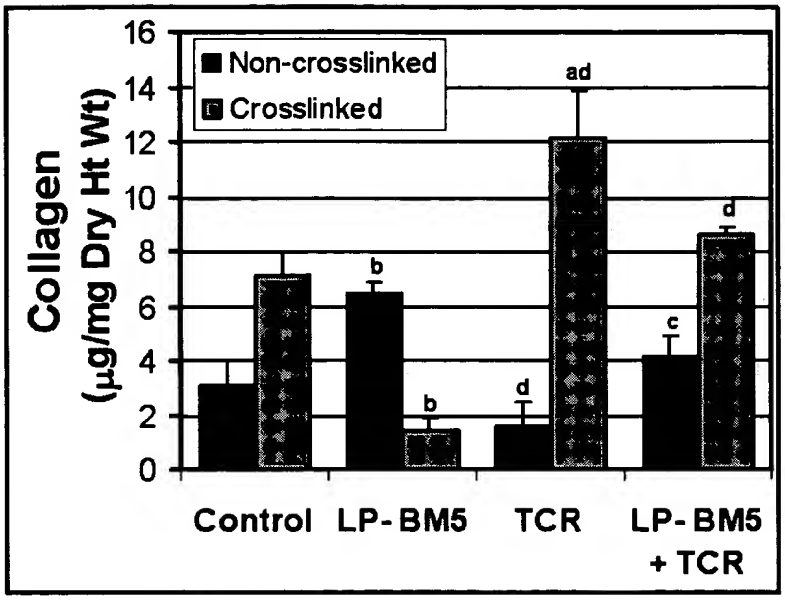

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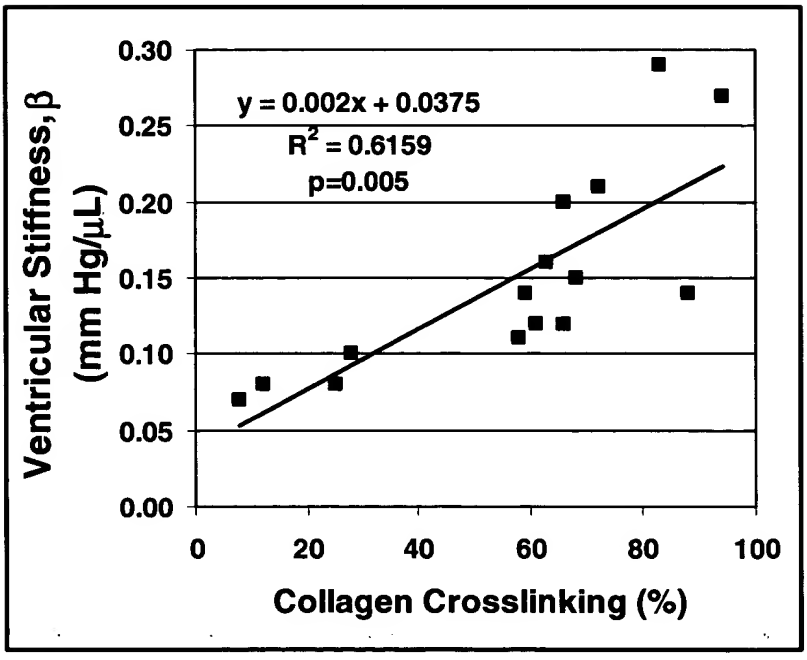

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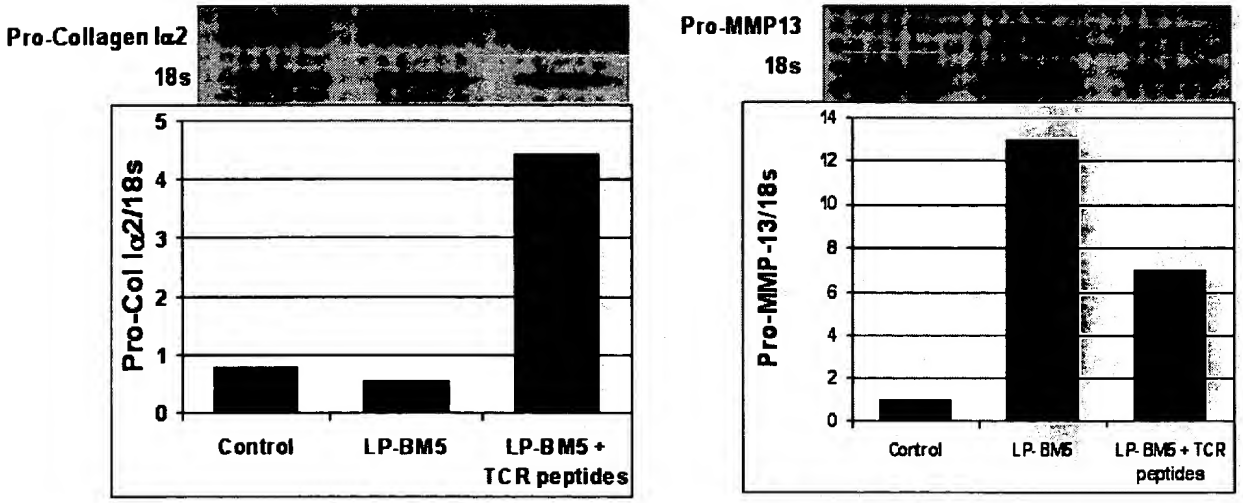

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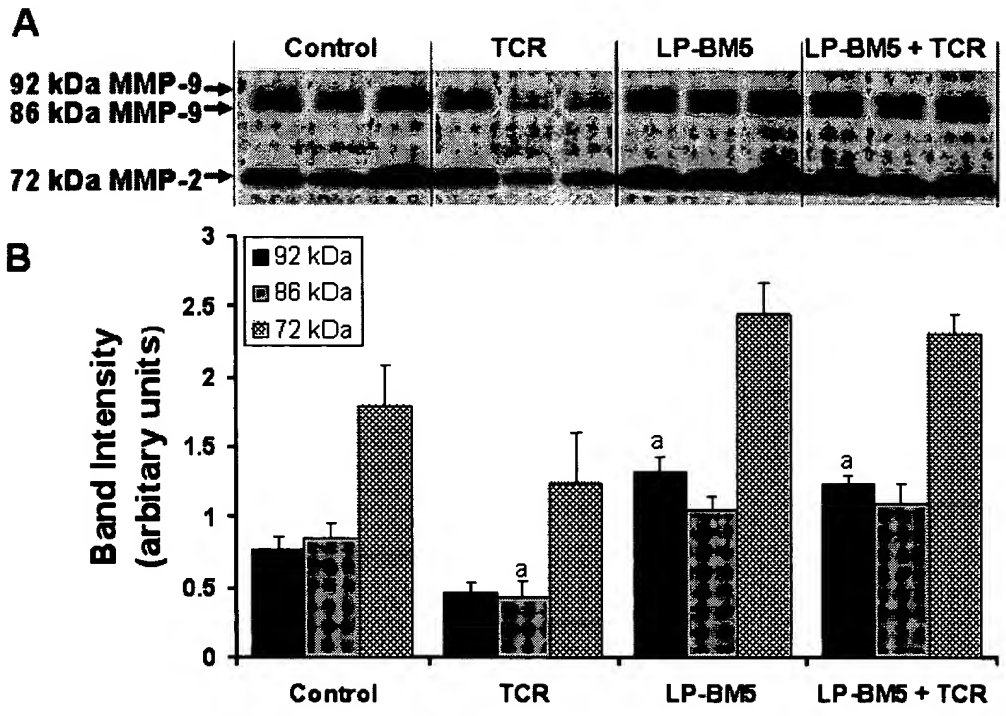

Legends

TABLE 2:

The effect of immune modulation on left ventricular hemodynamic function.

These data represent the mean ± SEM of cardiac functional parameters after 12 weeks of LP-BM5 infection or with TCR peptides Vβ 8.1 and 5.2 treatment (200mg/mouse) on days 14 and 28. HR = heart rate; SVI = stroke volume indexed with body weight; EF = ejection fraction; CI = cardiac output indexed with body weight; SWI = stroke work indexed with body weight: β = slope of end-diastolic pressure volume relationship plotted against Ved; Ved = end-diastolic volume; Ves = end-systolic volume; dP/dt_{max} = dP/dt maximum of isovolumic contraction; dP/dt_{min} = rate of diastolic relaxation; PRSW = preload recruitable stroke work = the slope of stroke work plotted against Ved; dP/dt_{max}-Ved = slope of dP/dt_{max} plotted against Ved describing isovolumic contraction; Tau weiss (τ) = time constant of isovolumic relaxation. Ea = arterial elastance.

[a] = $p < 0.05$ compared with control, [b] = $p < 0.01$ compared with control,

[c] = $p < 0.05$ compared with LP-BM5 infection, [d] = $p < 0.01$ compared with LP-BM5 infection.

Table 3.

The effect of immune modulation on splenic and cardiac gene expression.

(A) Real-time PCR analysis of splenic tissue three months after treatment with LP-BM5 and TCR peptide shows the relative induction of TH1 or TH2 cytokine gene expression. (B) The level of candidate cardiac genes for collagen, MMPs,

and LOXL3 were expressed as ratios to the control Group I gene expression using real-time PCR. The data are expressed as mean ± SEM.

[a] = $p < 0.05$ compared with control, [b] = $p < 0.01$ compared with control, [c] = $p < 0.05$ compared with LP-BM5 infection, [d] = $p < 0.01$ compared with LP-BM5.

Figure 1:

Characteristic *in vivo* pressure-volume loops of mice from the four treatment groups.

Figure 2:

The effect of immune modulation on total cardiac collagen. The apical portion of the left ventricle was assayed for hydroxyproline (HPRO) concentrations and normalized against dry weight of each sample. The HPRO concentrations were converted to relative collagen levels using the assumption that collagen contains an average of 13.5% HPRO. The data are expressed as mean ± SEM. [a] = $p < 0.05$ compared to control, [b] = $p < 0.01$ compared to control, [d] = $p < 0.01$ compared to LP-BM5

Figure 3:

The cardiac collagen crosslinking associated with immune modulation. The apical portion of the left ventricle was assayed for hydroxyproline (HPRO) concentrations; those that were insoluble to cyanogen bromide were considered crosslinked and HPRO concentrations that were soluble to cyanogen bromide

were regarded as non-crosslinked. The HPRO concentrations were converted to relative collagen levels using the assumption that collagen contains an average of 13.5% HPRO. The data are expressed as mean ± SEM. [a] = $p < 0.05$ compared with control, [b] = $p < 0.01$ compared with control, [c] = $p < 0.05$ compared with LP-BM5 infection, [d] = $p < 0.01$ compared with LP-BM5.

Figure 4: The relationship between cardiac collagen crosslinking and ventricular stiffness (β). The percentage of total crosslinked cardiac collagen was plotted against the *in vivo* measured ventricular stiffness. The correlation coefficient was $R^2 = 0.6156$ ($p = 0.005$), suggesting that crosslinking is a determinant of diastolic stiffness during the filling phase of diastole.

Figure 5: The effect of co-culturing lymphocytes with cardiac fibroblasts. Lymphocytes were harvested from the control, LP-BM5 only, and combined LP-BM5 plus TCR peptide mice 12 weeks after treatment and co-cultured with naive primary cardiac fibroblasts. Lymphocytes from three mice in each group were pooled and added to the cardiac fibroblast cultures at a concentration of 5 x 10^5/mL. After 48 h of co-incubation the lymphocytes were removed and the cardiac fibroblast RNA analyzed for *pro-collagen Iα2* and *pro-MMP-13* with RT-PCR. The graphed data is the ratio of the candidate gene versus 18s RNA.

Figure 6: The MMP activity of the supernatant from co-culturing lymphocytes with cardiac fibroblasts. Lymphocytes were harvested from the

control, TCR, LP-BM5, and combined LP-BM5 plus TCR peptide mice 12 weeks after treatment and were co-cultured with naïve primary cardiac fibroblasts. The MMP activity of the co-culture supernatants was assayed with zymography for the MMP-2 and -9 demonstrate activity. TH1 reduced MMP activity in vitro and TH2 enhanced MMP activity. [a] = $p < 0.05$ compared with control